(10)

AD

TECHNICAL REPORT 8305

THE ACUTE AND CHRONIC TOXICITY OF 3,5-DINITROANILINE, 1,3-DINITROBENZENE, AND 1,3,5-TRINITROBENZENE TO FRESHWATER AQUATIC ORGANISMS

WILLIAM H. van der SCHALIE, Ph.D.

US ARMY MEDICAL BIOENGINEERING RESEARCH and DEVELOPMENT LABORATORY
Fort Detrick
Frederick, MD 21701

OCTOBER 1983

Approved for public release;
distribution unlimited

US ARMY MEDICAL RESEARCH and DEVELOPMENT COMMAND
Fort Detrick
Frederick, MD 21701

NOTICE

Disclaimer

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

Disposition

Destroy this report when it is no longer needed. Do not return it to the originator.

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER TECHNICAL REPORT 8305	2. GOVT ACCESSION NO. AD A138408	3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) THE ACUTE AND CHRONIC TOXICITY OF 3,5-DINITROANILINE, 1,3-DINITROBENZENE, AND 1,3,5-TRINITROBENZENE TO FRESHWATER AQUATIC ORGANISMS		5. TYPE OF REPORT & PERIOD COVERED Technical Report Dec 1979 - Oct 1981 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) WILLIAM H. van der SCHALIE, Ph.D.		8. CONTRACT OR GRANT NUMBER(s)
9. PERFORMING ORGANIZATION NAME AND ADDRESS US Army Medical Bioengineering Research and Development Laboratory, ATTN: SGRD-UBG Fort Detrick, Frederick, MD 21701		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62720A 3E162720A835/00/125
11. CONTROLLING OFFICE NAME AND ADDRESS US Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick, Frederick, MD 21701		12. REPORT DATE October 1983 13. NUMBER OF PAGES 55
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) UNCLASSIFIED 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Acute toxicity	Channel catfish	Fathead minnow
Algae	Chronic toxicity	Fish
Aquatic toxicology	Daphnia magna	Invertebrates
Bluegill	Early life stage	Rainbow trout

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

The xicity to freshwater aquatic organisms of three compounds formed during the continuous manufacturing process for 2,4,6-trinitrotoluene (TNT) was determined. The compounds were 1,3-dinitrobenzene (DNB), 3,5-dinitroaniline (DiNA), and 1,3,5-trinitrobenzene (TNB). Four species of fish (fathead minnow, rainbow trout, channel catfish, and bluegill), one aquatic invertebrate (Daphnia magna), and one alga (Selenastrum capricornutum) were

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

UNCLASSIFIED

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

19. Key Words - Continued

Selenastrum capricornutum
1,3-Dinitrobenzene
1,3,5-Trinitrobenzene
3,5-Dinitroaniline

20. Abstract - Continued

tested. Emphasis was on determining the threshold levels of toxicity to the species found to be most sensitive to the test materials.

The 96-hour DNB LC50's for fish ranged from 1.4 to 16.8 mg/L. Daphnia magna was less sensitive than any of the fish, with a 48-hour EC50 (based on immobilization) of 27.4 mg/L. For rainbow trout (96-hour LC50 1.7 mg/L), chronic toxicity was estimated using a 69-day early life stage test. The range from the highest no-effect concentration to the lowest concentration causing toxicant-related effects was 0.50 to 0.97 mg/L. Juvenile trout tested in a 30-day exposure were even more sensitive, with a no effect-effect range for mortality of 0.16 to 0.42 mg/L. Selenastrum capricornutum was also relatively susceptible to DNB, with toxic effects on growth occurring at 0.97 mg/L (but not at 0.26 mg/L) in a 5-day static exposure test.

The static acute LC50's of DiNA for fish and D. magna ranged from 3.0 to 21.1 mg/L. The rainbow trout was most sensitive, and a 71-day early life stage test was conducted with this species. Toxicant-related effects were found at concentrations of 0.65 mg/L and above, but were not observed at 0.37 mg/L or less. Selenastrum capricornutum was the most sensitive of all species tested. Growth was inhibited at 0.13 mg/L (but not at 0.03 mg/L) during a 5-day static test.

TNB was quite toxic to fish; all LC50's were less than 1 mg/L (range 0.38 to 0.85 mg/L). The 48-hour EC50 for D. magna was higher (4.1 mg/L). A 21-day chronic test with this species revealed TNB toxicity at concentrations of 0.75 mg/L and above but not 0.47 mg/L or below. Early life stage tests were conducted with both fathead minnows (32 days) and rainbow trout (71 days). Toxic thresholds for these species were similar; no effects were observed at a concentration of 0.08 mg/L in both tests, but toxicity was evident at the next higher concentration--0.17 mg/L for rainbow trout and 0.12 mg/L for fathead minnows. A toxic threshold was not found for S. capricornutum, since growth reductions were observed at the lowest concentration tested (0.10 mg/L).

ACKNOWLEDGEMENTS

Thanks are due to many individuals and groups that made contributions to this project. The US Fish and Wildlife Service provided many of the fish used in the toxicity tests. Bluegills came from the Harrison Lake Fish Hatchery, while rainbow trout (both eggs and juveniles) came from the National Fisheries Center (Leetown, WV), the White Sulphur Springs National Fish Hatchery (White Sulphur Springs, WV), and the Wytheville National Fish Hatchery (Max Meadows, VA). Synthesis, purification, and toxicant concentration analyses were provided by the US Army Medical Bioengineering Research and Development Laboratory (USAMBRDL) chemists, including Dr. W.H. Dennis, Mr. A.B. Rosencrance, Mr. E.E. Brueggemann, and Ms. T. Trybus. 1LT S.G. Broich was instrumental to the conduct of many of the toxicity tests. Others making substantial contributions included Mr. T.R. Shedd, Mr. R.C. Bishoff, PFC M.M. Skarwecki, and SP5 E.C. Rawitz.

TABLE OF CONTENTS

TABLES

INTRODUCTION

The US Army Medical Research and Development Command has responsibility for assessing the possible health and environmental hazards associated with materials released during the production of munitions-related materials at Army ammunition plants. One such material is the explosive 2,4,6-trinitrotoluene (TNT). During the continuous manufacturing process for TNT, a "red water" waste is produced that is concentrated through a distillation process. The distillate, known as condensate wastewater, contains a large number of nitroaromatic by-products and has in the past received minimal treatment before being discharged into receiving bodies of water. The purpose of this investigation was to determine the toxicity to freshwater aquatic organisms of three components of condensate wastewater: 1,3-dinitrobenzene (DNB), 3,5-dinitroaniline (DiNA), and 1,3,5-trinitrobenzene (TNB). The latter two compounds are also formed in aquatic systems as by-products of the photolysis of TNT.[1] DNB is the third largest component of condensate wastewater, after 2,4- and 2,6-dinitrotoluene. Extensive work on the toxicity of 2,4-dinitrotoluene to aquatic organisms has already been conducted, along with some preliminary tests with 2,6-dinitrotoluene.[2]

Further investigations have explored the toxicity of an artificial 30-component condensate wastewater and the acute toxicity of each individual compound in the condensate mixture to aquatic organisms.[2] The results of these tests with DNB, DiNA, and TNB, along with other data from the literature on these three materials, are given in Table 1. From these rather limited data, DiNA would appear to be the least toxic of the compounds.

The present study utilized four fish species, one aquatic invertebrate, and one planktonic alga in an attempt to define the toxic thresholds of DiNA, DNB, and TNB to these aquatic organisms. Preliminary static acute tests with each species were followed by additional tests with the species found to be most sensitive to the toxic effects of each compound. These other evaluations included dynamic (flow-through) acute, early life stage (fish), and chronic (*Daphnia magna*) toxicity tests. The algal toxicity tests were done under contract, and the results were provided in a letter report.[3]

MATERIALS AND METHODS

TEST CHEMICALS AND ANALYTICAL CHEMISTRY

DNB and TNB were synthesized and purified by USAMBRDL chemists. DiNA was obtained from Aldrich Chemical Company (approximately 97 percent pure) and further purified by recrystallization from ethanol. The final purity of all test chemicals was in excess of 99.5 percent.

Analytical methods used for TNB, DNB, and DiNA are shown in Tables 2, 3, and 4, respectively. Blind spike samples, consisting of a known amount of toxicant dissolved in dilution water, were generally provided with each sample set taken during a test. Since average percent recoveries for these spike samples were nearly all between 90 and 100 percent, reported concentrations were not corrected for percent recovery.

TABLE 1. TOXICITY OF DINA, DNB, AND TNB TO AQUATIC ORGANISMS

Compound	Species	Period of Exposure	End Point	End Point Concentration mg/L (95% Confidence Limits)	Ref.
DINA	Water Flea (Daphnia magna)	48 hr	EC50	15.4 (13.5-18.0)	2
	Fathead Minnow (Pimephales promelas)	96 hr	LC50	21.8 (19.1-31.3)	2
DNB	Bluegreen Alga (Microcystis aeruginosa)	Not available	Threshold for inhibition of cell multiplication	0.17	6
	Green Alga (Scenedesmus quadricauda)	7 days	Threshold for inhibition of cell multiplication	0.7	7
	Protozoan (Entosiphon sulcatum)	72 hr	Threshold for inhibition of cell multiplication	0.76	7
	Fathead Minnow (Pimephales promelas)	96 hr	LC50	7.0 (5.8-8.1)	2
	Fathead Minnow (Pimephales promelas)	96 hr	LC50	12.7 (9.7-16.2)	10
	Redbelly Dace (Chrosomus sp.)	6 hr	Lethal threshold	8-10	8
	Golden Orfe (Lanciscus idus melanotus)	Not available	LC50	10	9
	Bacteria (Pseudomonas putida)	16 hr	Threshold for inhibition of cell multiplication	14	7
	Water Flea (Daphnia magna)	48 hr	LC50	49.6 (42.5-59.2)	2
TNB	Fathead Minnow (Pimephales promelas)	96 hr	LC50	1.1 (1.0-1.2)	2
	Water Flea (Daphnia magna)	48 hr	LC50	2.7 (2.4-3.1)	2

TABLE 2. ANALYTICAL CHEMISTRY METHODS FOR TNB

Toxicity Tests	Sample Extraction	Internal Standard	Instrument[a]	Column Conditions			Blind Spike Results		
				Length (cm)	Packing	Temperature	Average Percent Recovery	Range	n
Rainbow Trout, Bluegill Static Acute	100 mL sample into 1 mL methylene chloride; concentrate in Kaderna-Danish evaporator	Docosane	GC/FID	183	3% OV-1	220°C (isothermal)	94	86-101	3
Fathead Minnow, Channel Catfish, and Water Flea Static Acute; Rainbow Trout Dynamic Acute (one sample)	4 or 5 mL sample into 1 mL ethyl acetate	DINA	GC/NPD	183	3% OV-210	220°C (isothermal)	100[b]	87-113	5
Rainbow Trout Dynamic Acute (three samples)	Direct injection	None	HPLC	30	Reverse phase C_{18} μBondapak[c]		97	90-108	3
Rainbow Trout Early Life Stage Test	10 mL sample plus 1 mL saturated sodium sulfate into 2 mL ethyl acetate	DINA	GC/NPD	183	3% OV-210	195°C (isothermal)	96	63-140	10
Fathead Minnow Dynamic Acute and Early Life Stage	As above	DNB	GC/NPD	183	3% OV-210	220°C (isothermal)	92	82-98	7
Water Flea Chronic	As above	DINA	GC/NPD	183	3% OV-1	220°C (isothermal)	97[d]	87-105	3

a. GC - Hewlett-Packard Model 5830 gas chromatograph; NPD - nitrogen/phosphorus detector; FID - flame ionization detector; HPLC - Waters high performance liquid chromatograph.
b. One spike (28% recovery) excluded; sample preparation error suspected.
c. Other conditions: gradient - 45% methanol/55% water, 10 min; 2,500 psi; UV detector at 250 nm; 50 μL loop sample injection; flow rate 1.5 mL/min.
d. One spike (57% recovery) excluded; sample preparation error suspected.

TABLE 3. ANALYTICAL CHEMISTRY METHODS FOR DNB

Toxicity Tests	Sample Extraction	Internal Standard	Instrument[a]	Column Conditions			Blind Spike Results		
				Length (cm)	Packing	Temperature	Average Percent Recovery	Range	n
Water Flea, Fathead Minnow, Bluegill, and Rainbow Trout Static Acute Tests	4 mL sample into 1 mL ethyl acetate	TNB	GC/FID	183	3% OV-210	220°C (isothermal)	93	88-99	3
Channel Catfish Static Acute	1:1 with ethyl acetate	TNB	GC/FID	183	3% OV-210	220°C (isothermal)	95	-	1
Channel Catfish Static Acute	as above	TNB	GC/NPD	183	3% OV-210	175-210°C (20°/min)	72	65-80	2
Rainbow Trout Dynamic Acute	4 mL sample plus 1 mL ethyl acetate plus 1 mL internal standard solution	TNB	GC/NPD	183	3% OV-210	170-210° (20°/min)	90	69-100	4
Rainbow Trout Early Life Stage No. 1	10 mL sample plus 1 mL saturated sodium sulfate into 1 mL ethyl acetate	DiNA	GC/NPD[b]	183	3% OV-210	160°C (isothermal)	102[c]	86-115	10
Rainbow Trout Early Life Stage No. 2	As above, but used 2 mL ethyl acetate	TNT	GC/NPD	183	3% OV-1	200[d] (isothermal)	100	81-124	11

a. GC - Hewlett Packard Model 5830 Gas Chromatograph; NPD - nitrogen/phosphorus detector; FID - Flame ionization detector.
b. Peak height ratios rather than peak area ratios used for DNB quantification on three sample sets.
c. One spike (41% recovery) excluded; sample preparation error suspected.
d. 190°C (isothermal) for seven sample sets; peak height ratios used for DNB quantification in these samples.

TABLE 4. HPLC PARAMETERS FOR THE ANALYSIS OF DiNA

	Standard Conditions	Changes for Rainbow Trout Early Life Stage and Water Flea Chronic Tests
Column:	3.9 mm (ID) by 30 cm C_{18} μBondapak	
Mobile Phase:	65% methanol/35% water	63% methanol/37% water
Loop Size:	50 μL	100 μL
Detector:	UV, 250 nm	
Flow Rate:	1.5 mL/min	1.1 mL/min
Internal Standard:	None used; constant volume injections	
Retention Time:	3.91-4.03 min	
Sensitivity:	0.04	0.02
Pressure:	14 MPa	
Blind Spike Results		
Average percent Recovery	82	100[a]
Range	72-92	88-113
n	4	18

a. One spike (68% recovery) excluded; sample preparation error suspected.

In the fathead minnow static acute test, a gas chromatograph was used instead of the HPLC to measure test concentrations. A Hewlett Packard Model 5830 gas chromatograph was equipped with a nitrogen-phosphorus detector and a 183 cm by 2 mm (ID) column packed with 3 percent OV-210. The temperature was 220°C (isothermal), and the internal standard was TNB. Two blind spikes measured with this method had recoveries of 93 and 94 percent.

Analytical methods used in the algal toxicity tests have been described elsewhere.[4] Spike samples for these tests were made from a 1:20 dilution of the stock solution in algal assay medium. Percent recoveries for TNB, DiNA, and DNB were 85.2, 95.2, and 88.9, respectively.[5]

TOXICANT STOCK SOLUTIONS

Test solutions were prepared by diluting stock solutions of the test compounds. Stock solutions were made either in dilution water or in dimethylformamide (DMF). Stock solutions in water were made by adding excess toxicant to aerated well water, stirring for 24 to 72 hours, and filtering the solution to remove the remaining crystals. Distilled water was substituted for well water only if the stock solution made up less than about 1 percent of the test solution volume.

Stock solution concentrations were determined with a Beckman Acta V ultraviolet spectrophotometer. The solubilities of the three test compounds in well water at room temperature (20 to 23°C) were: TNB - 433 mg/L (range 397 to 461, n=8); DNB - 419 mg/L (range 371 to 463, n=7); and DiNA - 185 mg/L (range 166 to 196, n=6).

Stock solutions were prepared in DMF when extremely large quantities of aqueous stock solutions would otherwise have been required. All three test compounds dissolved readily in DMF. With TNB and DNB, DMF stock solutions were used only for the rainbow trout dynamic acute tests. DiNA was much less soluble in well water, so DMF was used as a carrier solvent in all tests except the rainbow trout static acute test and the daphnid tests.

When DMF was present, a control treatment with DMF present was included. Since DMF concentration increased with increasing toxicant concentration, the amount of DMF in this control was set equal to the amount of DMF present at the highest toxicant treatment level. The maximum concentration of DMF used was 0.5 mL/L in the static acute tests and 0.1 mL/L in flow-through tests.

DILUTION WATER QUALITY

Test solutions were made by mixing appropriate amounts of the stock solutions with dilution water. The dilution water was obtained from a 62-M well. Water was pumped through a spray nozzle (for aeration) into a 750-L reservoir tank. A second pump then sent the water through a 5-μ cellulose acetate cartridge filter, an ultraviolet sterilizer, and temperature adjustment equipment and then to the testing facility.

Because the well water is very hard, some precipitation of calcium carbonate was noted from fresh well water. To help prevent this, a water softening system was added to reduce the hardness of the water. For the tests listed in Table 5 (note a), flow reducers were used to deliver a mixture of 60 percent raw well water and 40 percent softened well water through the spray nozzle into the reservoir tank.

During the period of testing, weekly measurements were made of well water pH, hardness, alkalinity, total organic carbon, suspended solids, and ammonia. A summary of these data is given in Table 5. Test-specific measurements of pH, temperature, and dissolved oxygen concentration are discussed later in this report. More comprehensive analyses of well water are provided in Table 6. Nearly all the potentially toxic metals and organics were at nondetectable levels. The sporadic presence of DDD is an exception, but the concentration levels (maximum 40 ng/L in 1980) were extremely low. The relatively high levels of sodium in 1982 are related to the use of the water softener.

TABLE 5. SUMMARY OF WEEKLY DILUTION WATER QUALITY MEASUREMENTS

Parameter	Mean Value	Range	Number of Observations
pH	7.8	7.5-8.2	28
Hardness	290	228-339	38
(mg/L as $CaCO_3$)	177[a]	159-194	30
Alkalinity (to pH 4.5, mg/L as $CaCO_3$)	216	163-276	72
Conductivity (µmhos/cm)	660[b]	450-825	48
Total Organic Carbon (mg/L)	7.8	1-25	50
Suspended Solids (mg/L)	3.3	0-20[c]	60
Unionized Ammonia (µg/L)	<35		43

a. Includes data from these tests: rainbow trout early life stage tests with DiNA, DNB, and TNB; water flea chronic tests with DiNA and TNB.
b. During the rainbow trout early life stage test with TNB, the mean conductivity was 920 µmhos/cm (range 720-1250, n=10).
c. Few high values attributed to occasional flakes of carbonate material from water delivery lines.

The only fish or invertebrate culturing difficulty that seemed related to water quality was sporadic instances of neonate daphnids becoming caught in the surface film. These "floaters" never occurred in more than a few of the flow-through cultures at any one time. The problem was virtually eliminated by extra aeration of the well water and may have been related to a slight supersaturation of the water with gases, although dissolved oxygen concentration levels were always below the saturation point.

TEST ORGANISMS

Specific information on fish used in testing is given in Table 7. Fish were fed Rangens trout food pellets during holding. The higher loading levels were used successfully in the rainbow trout flow-through studies due to the greater turnover of test solution in these studies. Durotest Optima 50[R] * wide spectrum fluorescent bulbs (color rendering index of 91) were used with all organisms (including fish) during both holding and testing. The photoperiod was always 16 hours light and 8 hours dark.

* Use of trademarked name does not imply endorsement by the US Army, but is used only to assist in identification of a specific product.

TABLE 6. ANNUAL COMPREHENSIVE DILUTION WATER ANALYSES, 1980-1982

Parameter	Concentration (mg/L)			Chlorinated Hydrocarbons				
	1980	1981	1982[a]	Parameter	Concentration 1980	1981	1982	Detection Limit (µg/L)
Ammonia (as N)	0.33	0.49	0.03	Aldrin	X[b]	X	X	0.02
Nitrite (as N)	<0.01	—[c]	<0.02					
Nitrate (as N)	0.02	—	0.45	p,p'-DDT	X	X	X	0.02
Chloride	—	—	91.5	o,p-DDT	X	X	X	0.02
Fluoride	2.0	0.18	0.33	DDD	0.04	X	0.026	0.02
Sulfate	39.2	37.6	46.8	Dieldrin	T	X	X	0.02
Aluminum	0.047	<0.005	<0.002	Endrin	X	X	X	0.02
Barium	0.140	0.097	0.113	Heptachlor	X	X	X	0.02
Boron	<1.5	—	—	Heptachlor				
Cadmium	0.0004	<0.001	<0.001	Epoxide	X	X	X	0.02
Calcium	73.8	58.0	45.7	Lindane	X	X	X	0.01
Cobalt	<0.003	<0.005	<0.004	Chlordane	X	X	X	0.20
Copper	<0.02	<0.005	<0.003	Alpha-BHC	X	X	X	0.01
Iron	<0.03	<0.005	<0.003	Beta-BHC	T	-	X	0.02
Lead	<0.001	<0.002	<0.002	Delta-BHC	X	-	X	0.01
Magnesium	23.2	17.3	15.5	Toxaphene	X	X	X	1.0
Manganese	<0.02	<0.005	—	Methoxychlor	X	X	X	0.20[d]
Mercury	<0.001	<0.005	<0.0005	Polychlorinated				
Potassium	0.60	3.3	1.2	Biphenyls	-	<0.1	<0.001	—
Silicon	6.10	5.6	5.4					
Silver	<0.05	—	—					
Sodium	30.2	52.0	119.0					
Tin	<0.002	—	—					
Zinc	0.282	<0.005	<0.02					

a. Sample taken in 1982 was after switch from well water to 60% well water plus 40% softened well water.
b. X = below detection limit; T = trace (detectable peak, but below detection limit and not quantifiable). Concentrations reported in µg/L.
c. Not measured.
d. Detection limit 0.02 µg/L in 1982.

TABLE 7. INFORMATION ON FISH USED IN TESTING

Species	Source	Size Ranges Used in Testing[a]		Age[b] (weeks)	Length of Acclimation[b] (weeks)	Loading in Tests[b] (g fish/L test solution)	Acclimation Temperature (°C)	Diseases/ Treatment
		Standard Length (mm)	Wet Weight (g)					
Bluegill (*Lepomis macrochirus*)	Harrison Lake National Fish Hatchery, Harrison Lake, VA.	29–40	0.64–1.82	20–36	8–16	0.7–1.6	22±2	None
Channel Catfish (*Ictalurus punctatus*)	Blue Ridge Fish Hatchery, NC, and Kurtz Fish Hatchery, Elverson, PA	49–54	1.48–3.59	40–44	6–12	1.1–2.6	22±2	Malachite green plus formalin prior to DNB static acute test
Fathead Minnows (*Pimephales promelas*)	Fish: Kurtz Fish Hatchery, Elverson, PA Eggs: In-house culture unit	39–43	0.91–1.51	39–112	2–30	0.8–1.5	22±2[c]	None
Rainbow Trout (*Salmo gairdneri*, Wytheville strain)	Fish: National Fisheries Center, Leetown, WV[d]	35–40 (static acute tests)	0.67–0.95	10–13	4–7	0.7	12±2	None
		63–83 (flow through tests)	3.98–9.28	28–38	16–26	4.0–6.2	12±2	None

a. Range of test means reported.
b. Range for all tests.
c. 25±2°C for eggs.
d. Egg sources: National Fisheries Center, Leetown, WV; White Sulphur Springs National Fish Hatchery, White Sulphur Springs, WV (TNB and second DNB early life stage tests).

Daphnids (Daphnia magna) were raised in an in-house culture unit. Daphnids used in the static acute tests were obtained from stock cultures held in 19-L aquaria containing 15 L of well water. The temperature was 20°C (range 19 to 21), and the lighting had an average intensity of 625 lux (range 538 to 732). Dissolved oxygen levels were maintained by gently bubbling air through the cultures. Daphnids were fed a mixture of trout food (US Fish and Wildlife Service PR-11 formulation) and yeast at a concentration of 30 mg/L (dry weight) once per day (Monday through Friday), but only if food from the previous feeding had been mostly cleared from the water. Cultures were restarted in fresh well water every three weeks. Daphnids from cultures having ephippia, excessive mortality, or very low reproductive rates were not used in testing.

Daphnids used in chronic testing were raised differently. Stock daphnids were housed in 2-L tanks with 10 daphnids per tank. Aerated well water flowed through the tanks at a rate of two tank volumes per day. Temperature was maintained at 20°C (range 19 to 21), and light intensity was 150 to 350 lux. Daphnids were fed twice each day, seven days per week with vitamin enriched Ankistrodesmus falcatus using the methods of Goulden et al.[11] Feeding levels were approximately 2 mg/L (dry weight) in the morning and 4 mg/L in the afternoon. Young were removed from the tanks every Monday, Wednesday, and Friday.

TOXICANT DILUTER OPERATION

Flow-through toxicity tests were conducted using proportional diluters constructed as described in Lemke et al.[12] No neoprene stoppers were used, however, and glass-glass connections were made using only silicone glue or heat-shrinkable perfluorocarbon tubing. Toxicant stock solutions and, where appropriate, solvent control stock solutions were automatically delivered into the diluter system through Lab Industries Repipettes®, which were available in sizes capable of delivering from 0.01 to 50 mL per cycle. The Repipettes provided a calibrated amount of stock solution with each diluter cycle. Dilution water from the W1 chamber filled a bucket attached to a rod. The weight of the bucket forced the Repipette plunger down against a spring, delivering the toxicant into the M1 mixing chamber. The bucket then emptied through a self-starting siphon into the M1 chamber, and the Repipette was recharged with fresh toxicant solution as the rod returned to the up position. Daily measurements were made of stock solution usage rates and the cycle time of the diluter to ensure proper operation. Flow rates into the test tanks provided six tank volumes of test solution per day for fish dynamic acute and early life stage tests (diluters provided 0.5 L/treatment level/cycle) and two tank volumes per day for the daphnid tests (diluters provided 0.25 L/treatment level/cycle).

TEST END POINTS AND DATA ANALYSES

A fish was considered dead when ventilatory movements ceased and the fish failed to respond to gentle prodding. LC50s (and their 95 percent confidence limits) for mortality were determined using a computer program developed by C. Stephan.[13] The binomial method for estimating LC50s was used when there were less than two concentrations at which mortality was between 0 and 100 percent. Confidence limits generated with this method are actually greater than 95 percent (e.g. 97 or 99 percent); however, they are used here as conservative estimates of the 95 percent limits. Moving average or probit methods were used for determining LC50s when two or more responses between 0 and 100 percent occurred in a test. Probit results were recorded when the

goodness of fit probability of the data to the probit model was greater than 0.05. Moving average LC50 estimates were used if the probit goodness of fit was less than 0.05.

With daphnids, immobilization was used as an end point instead of death. Especially with daphnids exposed to DiNA, it was difficult to determine whether a daphnid was dead or immobilized without microscopic examination. The analysis of the mortality data was the same as with fish, except that an EC50 was calculated rather than an LC50.

The following end points were monitored during the fish early life stage tests: egg hatchability, days to 50 percent hatch, days to 50 percent swim-up (rainbow trout only, measured from the 50 percent hatch day), fry survival, overall survival, standard length and weight (measured at the end of the test), fry deformities (percent), and behavioral effects. Days to 50 percent hatch and swim-up and behavioral effects could not be analyzed statistically. When statistical differences were monitored, an initial chi square test for heterogeneity was applied to the data from replicate exposure tanks to determine if the replicates could be pooled. For the growth parameters, sample size was adjusted downward when heterogeneity was detected, according to the methods of Feder.[14]

For the count variables, including egg hatchability, fry survival, overall survival, and fry deformities, a multiple comparison statistical approach was used to determine which treatment groups were significantly different from the controls. (In one early life stage test, solvent controls were used in addition to water controls. Significant differences between the controls were not found for any parameter, so statistical comparisons were made using pooled water and solvent control groups.) A succession of 2 x 2 contingency table tests of homogeneity between each treatment group and the controls were done based on a one-tailed Fisher's exact test. Bonferroni's method was used to adjust for simultaneity. Growth measurements (standard length and weight) were analyzed using a one-way analysis of variance. Dunnetts multiple comparison procedure was used for detecting significant treatment effects as compared to the controls.

The following end points were monitored in daphnid chronic tests: immobilization, young per replicate tank, young per female per reproductive day (total young divided by the total days that the daphnids were alive after the onset of reproduction in the test tank), and growth (length from the apex of the head to the tip of the caudal spine). For these data, the General Linear Models program of the Statistical Analysis System[15] was used to generate a non-linear regression model (response vs. log [concentration]), from which concentrations eliciting a given response (e.g. an EC50) could be estimated. In this report, the results of pair-wise comparisons between treatments and the controls generated, using the General Linear Models program, are reported by indicating those treatments that produced significant differences ($p=0.05$) from the controls.

Growth, measured in terms of cells per milliliter, was monitored during the algal toxicity testing procedures. Significant differences in growth from the controls were evaluated after 5 and 14 days of exposure to toxicants. Five- and 14-day no-effect concentrations were determined using a one-way analysis of variance, with Williams' procedure[16] used for pair-wise tests for significant differences from the controls.

In addition, 5-day algistatic concentrations were determined. Algistatic toxicant concentrations are defined as those at which algal cell numbers were virtually unchanged from initial inoculum levels after 5 days of exposure, but which allowed logarithmic growth to resume over a 9-day period after the algae were transferred to growth medium that did not contain toxicant.[17] Toxicant treatments that prevented a return to logarithmic growth were considered to have been algicidal.

TEST METHODOLOGIES

Static Acute Tests

For both fish and daphnid tests, static acute methods generally followed those recommended by the American Society for Testing and Materials.[18] Fish to be used in testing were acclimated to the well water for the periods indicated in Table 7. Fish were not used in testing if they had any symptoms of disease within 10 days of the start of the test, or if more than 2 percent of the fish died within the 48 hours preceding the start of the test. Fish were transferred from stock tanks into holding tanks 48 hours prior to the start of a test and were not fed during this time. The fish were then randomly assigned to a test jar in groups of three to four.[19] There were three jars of 10 fish or two jars of 15 fish per treatment level, depending on the size of the fish. Test jars were randomly assigned to positions in the water bath.[19]

Fish were tested in 19-L glass jars containing 14 L of test solution. Four or five toxicant concentrations plus controls were used in each test. The concentrations were in a logarithmic series (ratios of 1.0, 1.8, 3.2, 5.6, 10.0), with the estimated LC50 at the "3.2" point. Temperature was held within one degree (Celsius) of the holding temperature (Table 7) by keeping the jars in a water bath. Water bath temperature was monitored with a 7-day temperature recorder, and temperatures were checked daily in one test jar. Lighting was of the same quality and photoperiod as was used during holding, but during some tests light intensity was higher. A maximum of 1900 lux was recorded during testing as compared to a maximum of 350 lux during holding. The pH and dissolved oxygen concentrations were measured after 0, 48, and 96 hours in one replicate of the control and in low, medium, and high toxicant concentrations. Aeration was initiated in all jars if the dissolved oxygen level fell below 40 percent of saturation at the test temperature. Other aspects of dilution water quality are summarized in Tables 5 and 6 and were discussed earlier.

Toxicant concentrations were measured, at a minimum, at the beginning and end of each test in the controls and the low, medium, and high concentrations. Mean measured concentrations at each treatment level were used in all data analysis procedures. When toxicant concentrations were not measured at all treatment levels, remaining nominal concentrations were multiplied by the average ratio of measured to nominal concentrations.

Loading levels (grams of fish per liter of solution) during the test are shown in Table 7. The levels are, in general, higher than the recommended 0.8 g/L.[18]

The testing approach used in daphnid static acute tests was, in many ways, identical to that used in the fish static acute tests. Only aspects unique to daphnid tests or different from the fish static acute tests are described.

Daphnids to be used in testing were obtained from females isolated from stock cultures less than 24 hours before the start of a test. Trout chow and yeast food was provided to these adults and their neonates up to the time that the young were pooled for testing. Neonates were transferred with an eyedropper having a fire-polished bore at least 2 mm in diameter. After pooling, daphnids were randomly assigned to positions in the testing water bath. Daphnids were moved in groups of two and three into 200 mL of test solution in 250 mL borosilicate glass beakers until each beaker contained five daphnids. Six beakers (30 daphnids) were used at each treatment level.

Test solutions were prepared in well water which had been aerated prior to use to ensure that dissolved oxygen levels were 90 to 100 percent of saturation. Samples of test solution for toxicant concentration measurement were taken at the beginning and end of the test. Test duration was 48 hours.

Dynamic Acute Tests

Dynamic (or flow-through) acute tests were conducted only with fish. The goal of these tests was to determine a long-term LC50 and to check for evidence of cumulative toxicity. The lengths of these tests varied from 10 to 30 days.

Fish were obtained and randomized to test tanks, and the test tanks were randomized into a water bath, as described for the static acute test procedures. Fish loading levels did not exceed recommended[18] levels of 10 g/L or 1 g/L/day. Fish were not fed for the 48 hours preceding a test, nor were they fed for the first 96 hours of the test in order to duplicate static acute test conditions. After 96 hours, fish were fed the same food as they had been given during acclimation once each day, ad libitum.

Fish were tested in 19-L aquaria which contained 15 L of test solution. The aquaria measured 40 cm by 20 cm by 25 cm with a drain hole at a height of 19 cm. Two replicate tanks with 15 fish per tank were used at each of five treatment levels. (Two replicates of 10 fish each were used in the TNB dynamic acute test with rainbow trout.) Controls and, when appropriate, solvent controls were included.

The pH and dissolved oxygen concentrations were monitored in one replicate of each treatment level every day. Water bath temperature was monitored continuously and was checked with a thermometer in two test tanks daily. Temperatures were maintained within one degree Celsius of the holding temperature in all tests except the rainbow trout test with DNB. In this test, a water chiller malfunction caused temperatures in the test tanks to rise to 14.7°C (the target was 12°C) on two of the 30 days of the test.

Dynamic acute test dissolved oxygen concentrations remained in excess of 60 percent of saturation, and aeration of the tanks was not required. The only exception was in the TNB dynamic acute test with fathead minnows, where a diluter malfunction caused a reduced flow of test solution to the tanks overnight and resulted in oxygen concentrations as low as 37 percent of saturation. No mortality (either immediate or delayed) could be associated with this incident.

Toxicant concentrations were determined analytically in at least one replicate of all treatment levels at the beginning of the test and weekly

thereafter. Mean measured concentrations were used in all data analysis procedures.

Fish Early Life Stage (ELS) Tests

These tests were conducted with both fathead minnows and rainbow trout. Procedures were similar to those recommended in draft test protocols developed by the American Society for Testing and Materials.[20]

The only fathead minnow ELS test conducted was with TNB. Eggs less than 24 hours old came from an in-house culture unit. Eggs were obtained from adult fathead minnows (10 to 11 months old) that had been held in aquaria containing spawning substrates (sections of 10 cm PVC pipe cut in half lengthwise). A polyethylene liner was placed in each substrate to facilitate removal of eggs. Spawning fathead minnows were fed frozen brine shrimp and Rangens No. 3 trout food and were kept at 25°C under light conditions similar to those described earlier for fish acclimation. Clean liners were placed into each substrate less than 24 hours prior to the start of the test. On the day of the test, about 450 eggs were removed from three different substrates by gently rolling the eggs with the tip of the finger. The eggs were pooled, then randomized[18] into beakers containing well water and egg cups. Eggs were transferred in groups of one or two; due to some clumping of the eggs, a few groups as large as five had to be used. The egg cups were 11.5 cm lengths of 50 mm ID glass tubing covered at the bottom with 508 micron polyethylene mesh screen.

Once the eggs were in the cups, the cups were transferred to the test tanks. At this point, each cup contained 35 eggs, and there were two replicate cups for each of the five treatment levels and the controls. Tanks were positioned randomly[19] in the water bath. A rocker-arm apparatus was used to keep the egg cups in motion. The vertical travel of the cups was about 2 cm at a speed of five cycles per minute. The average light intensity was 250 lux. Temperature was maintained at 25°C during the test; the actual range was 23.8 to 26.3°C. Dissolved oxygen concentrations and pH levels were measured in one replicate of each treatment level every day. Dissolved oxygen levels were between 7.9 and 8.9 mg/L, while pH levels ranged from 7.9 to 8.2. Toxicant concentrations were measured weekly in at least one replicate of each treatment level.

Each egg cup was examined daily, and dead eggs were removed from the beginning of the test until egg hatch was complete. When at least 90 percent of the eggs had hatched, the fry were released from the egg cup into the test tank. Any remaining eggs were left in the egg cup until they either hatched or died. Fry were fed less than 48-hour-old brine shrimp (Artemia salina) nauplii twice each day. Feedings were at least 6 hours apart. Excess food and fecal materials were siphoned from the tanks daily or as needed. Dead fry were counted and removed daily. Fry were not fed for the last 24 hours of the test to allow their guts to empty prior to weighing. The fathead minnow ELS test lasted 32 days.

For the rainbow trout ELS tests, eyed eggs were obtained from the sources indicated in Table 7. The eggs were changed from the temperature at which they were received to the test temperature (12°C) over a period of several hours. The eggs were then transferred in groups of 20 to egg baskets suspended in 19 L aquaria containing 15 L of test solution, until each egg basket

contained 60 eggs (80 eggs for the first test with DNB). With two replicate aquaria per treatment level, a total of 120 (160) eggs were used per treatment. The egg baskets were made from nylon mesh netting surrounding a stainless steel frame. The baskets measured 12 cm by 8 cm by 9 cm. They were kept in motion continuously by the same rocker arm apparatus described for the fathead minnow ELS test.

Due to the sensitivity of rainbow trout eggs to light, the eggs were shielded from light until 7 days after hatching. After this time, light intensity ranged from 250 to 350 lux. Temperature was maintained within a degree of 12°C, but in some ELS tests, temperatures as high as 14.5°C were encountered for short periods (less than 24 hours) due to cooling system difficulties. Dissolved oxygen concentrations averaged 8.5 to 9.0 mg/L, but when the levels dropped below 60 percent of saturation due to increased fish size (sometime between days 47 and 62 of the tests), aeration was begun in all tanks. The pH in all the tests averaged between 8.0 and 8.2 and ranged from 7.9 to 8.5. A carrier solvent (DMF) was necessary only in the ELS test with DiNA, and a solvent control was included which received an average of 0 089 mL/L DMF.

Egg hatching was recorded, and dead eggs were removed every day until hatching was complete. The trout were allowed to swim out of the egg basket when they reached the swim-up stage of development. Feeding was then initiated with standard US Fish and Wildlife Service formulation trout food. Fish were fed twice each day (at least six hours apart) on holidays and weekends and three times each day during the rest of the week. Food formulations were changed as appropriate to match the increasing size of the trout. Excess food and fecal materials were siphoned from the tanks daily.

The total duration of the rainbow trout ELS tests was at least 60 days following the 50 percent hatch day in the controls. Fish were not fed for the last 24 hours of the test to allow their guts to empty prior to weighing.

Daphnid Chronic Tests

In these tests, daphnids were exposed to the toxicants for 21 days. Four replicate tanks of 10 daphnids each were used at each treatment level. Neonates used to start the test were obtained from stock cultures of adults that were at least 21 days old. On the day of the test, less than 24-hour-old young were transferred into a beaker containing well water and algal food. Neonates from tanks containing any floating daphnids were not used. The young daphnids were then randomly[19] transferred to beakers containing test solution in a water bath held at the test temperature (20°C). Groups of three or four daphnids were transferred at a time using an eye dropper with an inside diameter greater than 2 mm and a fire-polished end.

After all the beakers contained 10 daphnids, the daphnids were introduced into the test tanks. The tanks were constructed of glass cemented with silicone sealer. Each tank was a 14-cm cube with a drain height of 10 cm and contained 2.3 L of test solution. The drain was covered with 286-μ polyethylene mesh screen to prevent the loss of daphnids from the tank. The drain was connected to an external standpipe which raised the solution level above the drain in the tank. This prevented daphnids from becoming stranded on the drain screen.

The toxicant diluter used in the daphnid chronic test functioned as described in the toxicant diluter section of this report. A total of 250 mL was delivered with each diluter cycle, which occurred every 20 minutes. This was split four ways (62.5 mL for each replicate tank).

Toxicant concentrations were measured weekly in at least one replicate of each treatment level. The dissolved oxygen and pH levels were measured in one replicate of each treatment every day. Water bath temperature was monitored continuously, and test tank temperature was checked in two tanks every day. Light intensity during testing was about 380 lux.

Daphnids were fed the same vitamin-enriched Ankistrodesmus falcatus that were provided to the stock cultures. Based on the dry weight per cell figures of Goulden et al.,[11] daphnids received 2 mg/L (dry weight) in the morning and afternoon in the TNB test. To increase overall young production, the afternoon feeding was increased to 4 mg/L in the DiNA chronic test.

Survival was noted daily in each test tank. After day 7 of the test, young production was monitored every Monday, Wednesday, and Friday. Adults were transferred to a beaker of test solution, and the test solution was then poured through a net which retained the neonate daphnids. The test solution was returned to the test tank along with the adults, and the neonates were rinsed into a beaker for counting. After 21 days, the length of each surviving daphnid was measured using a stereoscope with a calibrated ocular micrometer. Daphnids were immobilized prior to measurement by placing them in a solution containing one drop of Quinaldine in 10 mL acetone and 150 mL well water.

Algal Test Methods

Algal toxicity test procedures were based on those recommended by Payne and Hall.[17] Additional features, as described by Bailey,[3] are given here. Algae were exposed to toxicant solutions in 100 mL of algal assay medium contained in 500 mL Erlenmeyer flasks. Triplicate flasks were used at each treatment level except the controls, which had four replicate flasks. Each flask was inoculated with 20,000 cells/mL of Selenastrum capricornutum and held under 4,300 lux of cool white fluorescent light at a temperature of 26°C (range 25 to 27°C) for 14 days. Flasks were shaken continuously at 100 rpm on an orbital shaker table. Cell counts were made using an electronic particle counter on days 2, 5, 7, 9, 12, and 14. On day 5, those treatments showing anything less than a twofold increase in cell counts were pooled, centrifuged, and resuspended in triplicate flasks containing clean algal assay medium. All cultures were then grown for an additional 9 days.

Algal test solutions were sampled for toxicant concentration measurements at all treatment levels initially and on day 5 of exposure. For TNB, toxicant samples were taken only in the controls and in the low, medium, and high TNB concentrations on day 5 of the test. For the algal test results, only the initial concentrations are reported; no substantial loss of toxicant material from the test solutions was evident in any of the day 5 samples.

RESULTS AND DISCUSSION

3,5-DINITROANILINE

Results of the static acute tests with DiNA are shown in Table 8. At the higher concentrations, most of the fish and the daphnids, prior to death, were immobilized or had lower activity levels than the controls and the fish at lower concentrations. The low daphnid EC50 reported is somewhat misleading, since, as reported in Table 8, daphnids obtained from stocks fed algae instead of trout chow-yeast food were considerably more resistant to the effects of DiNA; the EC50 was higher by a factor of almost four. The higher EC50 is comparable to the 48 hour LC50 reported in Table 1 (15.4 mg/L). The fathead minnow 96-hour LC50s (Tables 8 and 1) were virtually identical.

TABLE 8. STATIC ACUTE TEST RESULTS WITH DiNA[a]

Species	End Point	LC/EC50, mg/L[b] (95% Confidence Limits)	Comments
Fathead Minnow	96 hr LC50	21.2 (15.1-29.9)[c]	d,e
Channel Catfish	96 hr LC50	13.9 (10.8-18.2)[c]	d,e
Bluegill	96 hr LC50	7.01 (3.92-14.4)[c]	d,e
Water Flea	48 hr EC50	3.76[f] (3.29-4.20)	
Rainbow Trout	96 hr LC50	2.99 (2.10-3.75)[c]	

a. Species arranged in order of increasing sensitivity.
b. Based on mean measured concentrations.
c. Binomial method used to compute LC50; confidence limits shown are actually greater than 95%.
d. Aeration used.
e. DMF used as a carrier solvent.
f. EC50 for daphnids obtained from adults fed algae instead of trout chow and yeast was 13.8 mg/L (12.9-14.8).

Additional, longer-term testing was conducted with the rainbow trout, which was the most sensitive species identified from these acute studies. In addition, daphnid chronic test results are reported since they are available from another study. In the dynamic acute test with rainbow trout, mortality ceased for 48 hours after day 9, and the test was terminated. At day 9, the LC50 was 1.95 mg/L with 95 percent confidence limits of 1.71 to 2.27. This LC50 was 35 percent below the 96-hour static acute LC50 (2.99 mg/L). The 1.95 mg/L figure is based on nominal concentrations determined from measured stock solution concentrations and toxicant and dilution water deliveries. Due to analytical difficulties, measurements of test solution concentrations could not be made.

The testing series with DiNA was continued with a rainbow trout ELS test. Concentration measurement data are given in Table 9. The test lasted 71 days (63 days post-hatch). Late in the test (days 54 and 57), the replicate tanks at the top concentration overflowed due to clogging of the drain screen, apparently due to excessive mucus production by the fish. No fish were lost because of these incidents.

TABLE 9. MEASURED DiNA CONCENTRATIONS IN THE RAINBOW TROUT EARLY LIFE STAGE TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
1.5	1.21	0.104	0.97-1.37	11
0.83	0.65	0.062	0.57-0.76	11
0.47	0.37	0.037	0.33-0.44	11
0.28	0.21	0.031	0.17-0.28	11
0.18	0.15	0.019	0.12-0.18	11
0 (DMF control)	BDL[a]	—	—	11
0 (water control)	BDL[a]	—	—	11

a. Below detection limit (0.069 mg/L).

The results of the ELS test are given in Table 10. Significant effects were seen at the top two concentrations. The effect on overall survival at 0.21 mg/L is of marginal statistical significance ($p=0.04$) and is not reflected in significant effects on either hatching success or fry survival. Effects were observed at 0.65 mg/L on hatching success (4.6 percent below control levels) and overall survival (~7.5 percent). The no effect level for DiNA and rainbow trout is estimated to lie between 0.37 and 0.65 mg/L.

The toxicity of DiNA to daphnids was explored in conjunction with another research project, so chronic toxicity data are available even though Daphnia magna was not one of the more sensitive species tested (Table 8). The concentrations tested are given in Table 11. As shown in Table 12, there was a cutoff in toxicity between 2.41 and 4.56 mg/L. At 4.56 mg/L, significant effects were noted for length (10 percent below controls), young per female per reproductive day (-21 percent), and total young per test tank of 10 daphnids (-20 percent). At 7.98 mg/L, young first appeared in the brood chambers on day 6 or 7 as compared to day 5 for all other daphnids. Also at this concentration, undeveloped young were released at times during the test; this was not seen at other treatment levels.

TABLE 10. RESULTS OF AN EARLY LIFE STAGE TEST WITH RAINBOW TROUT AND DINA

Mean Measured Concentration (mg/L)	Hatching Success (%)	Time to Hatch (days)[a]	Time to Swim-Up (days)[a]	Fry Survival (%)	Overall Survival (%)	Fry Deformities (%)	Fry Length (mm)	Fry Weight (g)
1.21	93.3[c]	25	18.0	74.1[d]	69.2[d]	1.8	40.5[c]	1.06
0.65	93.3[c]	24.5	17.5	91.1	85.0[c]	1.8	43.7	1.34
0.37	94.2	24.5	17	92.0	86.7	2.7	44.4	1.39
0.21	95.0	25	16	90.4	85.8[c]	0	45.4	1.46
0.15	96.7	25	16.5	94.8	90.9	0	43.4	1.26
<0.07 (solvent control)	98.3	25.5	15.5	94.9[b]	93.3[b]	0.8	43.2	1.24
<0.07 (water control)	97.5	25	16	94.0	91.6	1.7	43.8	1.29

a. Not analyzed statistically.
b. Tank to tank heterogeneity detected.
c. Significantly different from the controls at the $p < 0.05$ level.
d. Significantly different from the controls at the $p < 0.01$ level.

TABLE 11. MEASURED DiNA CONCENTRATIONS IN THE DAPHNID CHRONIC TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
10.9	7.98	0.221	7.76-8.32	8
5.9	4.56	0.044	4.49-4.60	8
3.2	2.41	0.044	2.34-2.47	8
1.7	1.29	0.054	1.20-1.32	8
0	BDL[a]	--	--	4

a. BDL - Below detection limit (0.34 mg/L).

The presence of daphnids caught in the water surface film was noted and is recorded in Table 12. There seems to be a trend towards more floating daphnids at higher concentrations, although some floating was also seen in the controls. Another apparent trend is that some daphnids at the lower concentrations and the controls had the distal portions of their second antennae missing. Daphnids at the higher concentrations were quite lethargic; if the damage to the antennae were somehow related to movement, a lower occurrence of damage at higher concentrations would be expected.

Data from the DiNA algal toxicity test are presented in Table 13. Concentrations of 0.13 mg/L and above decreased algal growth relative to the controls after 5 days of exposure. The algae at the top three concentrations, which were transferred to clean media after 5 days, showed renewed growth during the last 9 days of the test. Thus, DiNA concentrations of 0.13 to 15.1 mg/L are considered to be algistatic, while the no observed effect concentration lies between 0.03 and 0.13 mg/L. Selenastrum capricornutum was the most sensitive of all the organisms exposed to DiNA.

1,3-DINITROBENZENE

There was a fairly large range in the sensitivity of the organisms tested to DNB (Table 14). Daphnids were the least sensitive, and as such were not retested with algal-fed daphnids as was done with the daphnids exposed to DiNA. However, literature toxicity data for DNB and daphnids (Table 1) show a 48-hour LC50 nearly twice as high as the EC50 reported in Table 14. The fathead minnow 96 hour LC50 (16.8 mg/L) compares favorably with one literature value (12.7 mg/L), but not with the other (7.0 mg/L), as shown in Table 1.

There was a difference of almost a factor of 20 between the daphnid EC50 and the LC50s of the most sensitive fish (bluegills and rainbow trout). The LC50s for these fish were also substantially below those for the redbelly dace and the golden orfe (Table 1). As evidenced by the high control mortality, the channel catfish were not in the best of condition for testing, but, since they were not among the most sensitive species, the test was not repeated.

TABLE 12. RESULTS OF A CHRONIC TOXICITY TEST WITH DAPHNIA MAGNA AND DINA

Mean Measured Concentration (mg/L)	Survival (%)	Young Per Female Per Reproductive Day	Total Young Per Tank	Total Length (mm)	Daphnids With Distal Portions of Antennae Missing	Floater Days[a,b]
7.98	88.5	2.97[c]	321[c]	4.20[c]	0	62
4.56	100.0	7.00[c]	980[c]	4.78[c]	0	13
2.41	90.0	9.18	1201	5.06	12	4
1.29	92.5	9.92	1314	5.23	23	4
<0.34 (control)	97.5	8.91	1230	5.30	15	8

a. Not analyzed statistically.
b. Floater days - The sum of the number of F0 daphnids observed caught in the surface film each day at each treatment level over the duration of the test.
c. Significantly different from the controls at the $p < 0.01$ level.

TABLE 13. EFFECTS OF DINA ON THE GROWTH OF SELENASTRUM CAPRICORNUTUM[a]

Initial Measured Concentration (mg/L)	Growth (cells/mL)[b] Day 5	Day 14
15.13[c]	27,840[d]	359,715[d]
8.74[c]	36,889[d]	1,554,364[d]
1.46[c]	24,142[d]	564,889[d]
0.82	293,833[d]	2,850,987
0.13	729,910[d]	1,999,458
0.03	2,123,164	2,964,142
Control	2,387,153	3,903,667

a. Data from Bailey.[3,5]
b. Mean of three replicates (four for controls).
c. Cells washed and resuspended in clean media on Day 5.
d. Significantly different from the controls at the $p < 0.05$ level.

TABLE 14. STATIC ACUTE TEST RESULTS WITH DNB[a]

Species	End Point	LC/EC50, mg/L[b] (95% confidence limits)	Comments
Water Flea	48 hr EC50	27.4 (24.0-31.4)	
Fathead Minnow	96 hr LC50	16.8 (11.4- +∞)	c,e
Channel Catfish	96 hr LC50	8.13 (7.10-9.30)	d,e
Rainbow Trout	96 hr LC50	1.70 (1.39-2.43)	c
Bluegill	96 hr LC50	1.44 (1.20-2.30)	c,e

a. Species arranged in order of increasing sensitivity.
b. Based on mean measured concentrations.
c. Binomial method used to compute LC50; confidence limits shown are actually greater than 95%.
d. Best available data; mortality in three control replicates was 0/10, 0/10, and 6/10. No similar disparity in mortality between replicates was seen in the other treatments.
e. Aeration used.

Further testing was conducted with the rainbow trout. Although the bluegill was slightly more sensitive in terms of the acute LC50s, the overlap of confidence limits indicated that the difference was not significant. The trout was selected for additional testing primarily due to the potential difficulties anticipated in performing early life stage tests with bluegills.

A dynamic acute test conducted with rainbow trout turned out to be a fairly long-term test. Concentration data are reported in Table 15. Mortality was over 20 percent at the top concentration (3.00 mg/L) after 4 days, at the next concentration (1.42 mg/L) after 8 days, at 0.76 mg/L after 10 days, and at 0.48 mg/L after 20 days. No mortality was seen at the lowest concentration tested (0.16 mg/L) or in the water or solvent controls. The test was terminated after 30 days; the LC50 at this time was 0.37 mg/L (95 percent confidence limits approximately 0.16 to 0.76 mg/L as determined by the binomial method). Fish lost equilibrium prior to dying. The 30-day LC50 was nearly five times lower than the 96-hr static acute LC50.

In view of the pattern of continuing mortality with rainbow trout and DNB, a 68-day (60 days post-hatch) ELS test was conducted. Concentration data are given in Table 16, while results are shown in Table 17. Nearly all fish at the top concentration had erratic swimming patterns and showed a loss of equilibrium. At the next lower concentration (0.44 mg/L), similar behavior was seen in two or three fish. The survival heterogeneity seen at 0.27 and 0.08 mg/L resulted from tank overflows in one replicate tank at each of these concentrations; overflows also occurred at the top concentration. These overflows resulted from clogging of the tank drains, apparently due to excessive mucus materials secreted by the fish. Some fish were lost from the tanks during these overflows; but, since it was not possible to determine exactly how many were lost or from what tanks the fish came, any fry missing at the end of the test were recorded as dead.

The results of the test are probably not greatly affected by the tank overflows. The only significant differences in survival were found at the top concentration, and these were apparent long before the tank overflows, which did not occur at the top concentration until days 66 and 67 of the 68-day test. The only other toxicant effects observed were reductions in length (13 percent below controls) and weight (25 percent below controls) at the top concentration. The significant increase in weight found at 0.27 mg/L is anomolous and cannot be readily explained. Based on the survival and growth results and the loss of equilibrium of fish at the top DNB concentration, the lowest effect and highest no-effect concentrations are 0.84 mg/L and 0.44 mg/L, respectively.

Although these conclusions regarding the DNB ELS test are most probably valid, the uncertainty created by the overflow of tanks during the test was sufficient to cause the test to be redone. Test concentrations used in this second test are given in Table 18, while results are given in Table 19. This test lasted 69 days (60 days post-hatch). Erratic swimming by fish at the top concentration was first noticed on day 34 of the test. By day 62, all fish at the top concentration were affected as were three to four fish at 0.50 mg/L. Statistically significant effects seen at the top concentration include reductions in fry survival, overall survival, length (14 percent lower than controls), and weight (38 percent below controls). The effect at 0.50 mg/L on fry survival had only marginal statistical significance; no effect on overall survival was found.

TABLE 15. MEASURED DNB CONCENTRATIONS IN A RAINBOW TROUT DYNAMIC ACUTE TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
2.65	3.00	0.454	2.52-4.26[a]	12
1.44	1.42	0.102	1.28-1.57	12
0.79	0.76	0.067	0.63-0.84	12
0.48	0.42	0.051	0.37-0.55	11
0.16	0.16	0.036	0.12-0.22	9
0 (DMF control)	BDL[b]	—	—	12
0 (water control)	BDL[b]	—	—	12

a. The 4.26 mg/L concentration is considered to be an outlier value; the corresponding concentration in the other replicate tank was 3.11 mg/L.
b. BDL - Below detection limit (0.1 mg/L).

TABLE 16. MEASURED DNB CONCENTRATIONS IN THE FIRST RAINBOW TROUT EARLY LIFE STAGE TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
0.84	0.84	0.074	0.64-1.00	22
0.45	0.44	0.035	0.39-0.54	22
0.26	0.27	0.024	0.21-0.32	22
0.15	0.14	0.032	0.07-0.24	22
0.08	0.08	0.021	0.02-0.11	21
0	BDL[a]	—	—	22

a. BDL - Below detection limit (0.02 mg/L).

TABLE 17. RESULTS OF AN EARLY LIFE STAGE TEST WITH RAINBOW TROUT AND DNB

Mean Measured Concentration (mg/L)	Hatching Success (%)	Time to Hatch (Days)[a]	Time to Swim-Up (Days)[a]	Fry Survival (%)	Overall Survival (%)	Fry Deformities (%)	Fry Length (mm)	Fry Weight (g)
0.84	97.5	28	17	50.6[b]	49.4[b]	2.6	31.5[c]	0.53[c]
0.44	100.0	28.5	18	74.4	74.4	3.1	36.0	0.69
0.27	96.9	28	18	79.4[d]	76.9[d]	3.2	37.0	0.78[c,e]
0.14	98.1	28	17.5	74.4	74.4	3.1	36.6	0.69
0.08	96.8	28	17	74.8[d]	72.5[d]	3.2	36.9	0.71
<0.02 (control)	98.1	28	16.5	75.8	74.4	3.2	36.1	0.71

a. Not analyzed statistically.
b. Significantly different from the controls at the $p < 0.01$ level.
c. Significantly different from the controls at the $p < 0.05$ level.
d. Tank to tank heterogeneity detected.
e. Weight statistically greater than the controls ($p < 0.05$).

TABLE 18. MEASURED DNB CONCENTRATIONS IN THE SECOND RAINBOW TROUT EARLY LIFE STAGE TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
1.00	0.97	0.096	0.71-1.05	11
0.57	0.50	0.035	0.44-0.54	11
0.33	0.32	0.017	0.29-0.35	10
0.17	0.16	0.019	0.12-0.19	11
0.11	0.10	0.012	0.08-0.13	11
0	BDL[a]	—	—	11

a. BDL - Below detection limit (0.04 mg/L).

Mucus production by trout was excessive in the top two concentrations of DNB. This again caused tank overflows on days 58 and 65 at the top concentration and on day 63 in one replicate of the next lower concentration. However, discrepancies between fish counts during the test and the number of fish measured at the end of the test were minimal. Also, survival was drastically affected by DNB at the top concentration well before any overflow occurred.

The results of both rainbow trout ELS tests with DNB are quite consistent. Using the results of the second test, which had a minimal problem with tank overflow, the effect/no effect levels for DNB are 0.97 and 0.50 mg/L, respectively. It is quite interesting that the 30-day LC50 for juvenile rainbow trout is actually lower (0.37 mg/L) than the levels of DNB found to be toxic to eggs and fry. With most compounds, the older fish are more resistant to toxicant effects.[21] Although it is possible that the presence of DMF increased DNB toxicity in the dynamic acute test, no mortality was seen in the DMF controls. Since 60 percent of fish exposed to 0.42 mg/L DNB in the 30-day test were killed, while no fish died at the next lower concentration (0.16 mg/L), the lowest no effect/effect range for rainbow trout exposed to DNB is 0.16 to 0.42 mg/L.

The alga Selenastrum capricornutum was about as sensitive to the effects of DNB as the most sensitive of the fish species tested. As Table 20 shows, 5-day growth of the algae was reduced at all concentrations above 0.26 mg/L. The no effect/effect concentration range (0.26 to 0.97 mg/L) includes the thresholds for inhibition of cell multiplication by DNB reported for two other algal species in Table 1.

TABLE 19. RESULTS OF THE SECOND EARLY LIFE STAGE TEST WITH RAINBOW TROUT AND DNB

Mean Measured Concentration (mg/L)	Hatching Success (%)	Time to Hatch (days)[a]	Time to Swim-Up (days)[a]	Fry Survival (%)	Overall Survival (%)	Fry Deformities (%)	Fry Length (mm)	Fry Weight (g)
0.97	98.3	24	16.5	27.1[b,c]	26.7[c]	3.4	35.9[d]	0.68[d]
0.50	97.5	25	16	83.6[b,d]	80.8	1.7	41.3	1.09
0.32	90.0	25	15	94.4	85.0	0	43.1	1.27
0.16	96.7	24.5	16	96.6	93.3	0.9	43.2	1.25
0.10	95.0	25	15	92.0	86.7	0	42.9	1.25
<0.04 (control)	88.3	24.5	15.5	92.2	88.3	0	41.6	1.10

a. Not analyzed statistically.
b. Tank to tank heterogeneity detected.
c. Significantly different from the controls at the $p < 0.01$ level.
d. Significantly different from the controls at the $p < 0.05$ level.

TABLE 20. EFFECTS OF DNB ON THE GROWTH OF SELENASTRUM CAPRICORNUTUM[a]

Initial Measured Concentration (mg/L)	Growth (cells/mL)[b] Day 5	Day 14
85.63[c]	29,529[d]	24,969[d]
14.33[c]	38,498[d]	2,672,764[d]
10.72[c]	36,711[d]	2,758,560[d]
1.58	76,418[d]	376,044[d]
0.97	207,938[d]	3,840,755
0.26	3,092,720	5,582,044
Control	3,446,286	6,316,560

a. Data from Bailey.[3,5]
b. Mean of three replicates (four for controls).
c. Cells washed and resuspended in clean media on Day 5.
d. Significantly different from the controls at the $p < 0.05$ level.

Selenastrum capricornutum exposed to 10.7, 14.3, and 85.6 mg/L DNB were resuspended in clean media after 5 days, and all but those algae previously exposed to 85.6 mg/L showed renewed growth. The 85.6 mg/L exposure level is therefore considered to be algicidal, while concentrations between 0.97 and 14.3 mg/L appear to be algistatic.

1,3,5-TRINITROBENZENE

Overall, TNB was the most toxic of the three compounds tested. The static acute tests (Table 21) resulted in LC50s for fish that were all less than 1 mg/L. The daphnids were somewhat less sensitive, and algal-fed daphnids were still less, with 48-hour EC50s in the range of 4 to 6 mg/L.[22] The literature LC50 for D. magna was intermediate to these, while the literature LC50 for fathead minnows was twice the one in Table 8 (see Table 1).

Due to the closeness of the fish LC50s and the considerable overlap in the confidence intervals, testing was continued with the two fish species for which ELS testing methods were best developed: fathead minnows and rainbow trout. Dynamic acute tests were conducted prior to the early life stage tests with each species. With fathead minnows, a 10-day LC50 of 0.46 mg/L was obtained (95 percent confidence limits 0.42 to 0.53). Concentration data are reported in Table 22. Mortality was still continuing after 10 days, but a diluter malfunction forced termination of the test at that time. Low dissolved oxygen concentrations (as low as 3.2 mg/L) were noted on day 9, due to a temporary shutdown of the diluter. This problem was quickly corrected and did not appear to adversely affect the fish.

TABLE 21. STATIC ACUTE TEST RESULTS WITH TNB[a]

Species	End Point	LC/EC50, mg/L[b] (95% Confidence Limits)	Comments
Water Flea	48 hr EC50	2.98 (2.63-3.38)	
Bluegill	96 hr LC50	0.85 (0.52-1.38)	c,d
Rainbow Trout	96 hr LC50	0.52 (0.37-0.80)	c,e
Fathead Minnow	96 hr LC50	0.49 (0.44-0.56)	d
Channel Catfish	96 hr LC50	0.38 (0.34-0.43)	d

a. Species arranged in order of increasing sensitivity.
b. Based on mean measured concentration.
c. Binomial method used to compute LC50; confidence limits shown are greater than 95%.
d. Aeration used.
e. Initial toxicant sample set lost; LC50 based on average of nominal initial concentration and 96 hour measured concentration.

TABLE 22. MEASURED TNB CONCENTRATIONS IN A FATHEAD MINNOW DYNAMIC ACUTE TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
1.42	1.21	0.244	0.99-1.47	5
0.78	0.45	0.026	0.42-0.48	4
0.43	0.32	0.024	0.28-0.35	5
0.26	0.20	0.006	0.18-0.21	5
0.14	0.12	0.070	BDL[a]-0.19[b]	5
0	BDL[a]	—	—	4

a. BDL - Below detection limit (0.05 mg/L).
b. One set of measurements was quite different between the two replicate tanks (BDL and 0.19 mg/L).

The rainbow trout dynamic acute test was stopped after 18 days, since mortality over the last 4 days of the test was low (two additional deaths out of 20 exposed fish at one concentration only, 0.37 mg/L). The 18-day LC50 was 0.43 mg/L (0.24 to 0.73 - 95 percent confidence limits by the binomial method). The 10-day LC50 (0.52 mg/L, binomial confidence limits 0.37 to 0.73) was quite close to the fathead minnow 4 day LC50 (Table 21). Concentration measurement data for this test are reported in Table 23.

TABLE 23. MEASURED TNB CONCENTRATIONS IN A RAINBOW TROUT DYNAMIC ACUTE TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
1.44	1.42	0.085	1.32-1.54	6
0.78	0.73	0.056	0.69-0.84	6
0.41	0.37	0.038	0.30-0.41	6
0.25	0.23	0.028	0.18-0.26	6
0.11	0.06[a]	—	—	2
0 (solvent control)	BDL[b]	—	—	6
0 (water control)	BDL	—	—	6

a. Detection limit was 0.10 mg/L; this value was estimated based on one sample set that could be quantified.
b. BDL - Below detection limit (0.10 mg/L).

Early life stage tests were conducted for both fathead minnows and rainbow trout. The exposure concentrations for the fathead minnow test are shown in Table 24, while the test results are given in Table 25. Significant reductions in both fry and overall survival were noted at all but the lowest concentration tested. The reduction in length at 0.08 mg/L is thought to be an artifact since no corresponding weight reductions were found and no significant length effects occurred at higher concentrations. The increased weights at higher concentration levels may be related to the lower density of fish due to TNB-related mortality. One behavioral effect was noted: fry at all concentrations appeared to be less active than the controls.

TABLE 24. MEASURED TNB CONCENTRATIONS IN A FATHEAD MINNOW EARLY LIFE STAGE TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
0.83	0.72	0.084	0.61-0.89	11
0.46	0.32	0.028	0.28-0.37	11
0.25	0.18	0.013	0.16-0.20	11
0.15	0.12	0.017	0.09-0.15	11
0.11	0.08	0.017	0.06-0.11	11
0	BDL[a]	—	—	10

a. BDL -Below detection limit (0.05 mg/L).

Rainbow trout appeared to have approximately the same sensitivity as fathead minnows to TNB in an ELS test. The initial rainbow trout ELS test had to be repeated because significant effects were observed at the lowest concentrations tested. The concentrations tested and the test results for the first test are recorded in Tables 26 and 27, respectively. The test lasted 68 days (60 days post-hatch). Three fish were accidently lost during the test (one at 0.22 mg/L and two from either the control or 0.09 mg/L). These fish were counted as dead at the end of the test for data analysis purposes. Significant effects at the lowest concentration tested included an average 26 percent reduction in weight and an average 11 percent reduction in length from the controls.

The second early life stage test with rainbow trout and TNB lasted 71 days (61 days post-hatch). TNB concentrations tested ranged from 0.015 to 0.17 mg/L (Table 28). There was considerable overlap between the bottom two concentrations, but, since no significant effects were noted except at the top concentration (Table 29), this does not affect the interpretation of the test results. Behavioral effects were noted at both 0.17 and 0.082 mg/L. Fish at these concentrations were lighter in color than the controls, exhibited erratic swimming patterns, and, at 0.17 mg/L alone, had copious mucus production.

A comparison between the results of the two ELS tests with rainbow trout and TNB (Table 30) shows a fairly high degree of consistency. Corresponding concentrations resulted in approximately the same degree of effect, except that trout in the first test may have been slightly more sensitive in terms of length and weight effects at the lowest concentration (0.09 mg/L). Based on these data, the no effect/effect concentration range is 0.082 to 0.17 mg/L.

TABLE 25. FATHEAD MINNOW EARLY LIFE STAGE TEST RESULTS

Mean Measured Concentration (mg/L)	Time to Hatch (Mean Days to 50% Hatch)	Hatching Success (%)	Fry Survival (%)	Overall Survival (%)	Fry Deformities (%)	Fry Length (mm)	Fry Weight (mg)
0.72	4	97.1	0[a]	0[a]	2.9	—	—
0.32	4	95.7	26.9[a]	25.7[a]	0	12.6	36
0.18	3.5	91.4	65.6[a]	60.0[a]	1.6	13.2	34[b]
0.12	3	94.3	69.7[a]	65.7[a]	0	13.2	31
0.08	3	98.6	82.6	81.4	1.4	12.7[b]	30
<0.05 (control)	3.5	95.7	92.5	88.6	1.5	13.4	29

a. Significantly different from the controls at the $p < 0.01$ level.
b. Significantly different from the controls at the $p < 0.05$ level.

TABLE 26. MEASURED CONCENTRATIONS IN THE FIRST RAINBOW TROUT EARLY LIFE STAGE TEST WITH TNB

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
0.75	0.71	0.066	0.60–0.82	13
0.41	0.36	0.051	0.27–0.44	18
0.23	0.22	0.053	0.15–0.35	20
0.14	0.13	0.037	0.07–0.21	20
0.09	0.09	0.019	0.06–0.12	17
0	BDL[a]	—	—	20

a. BDL - Below detection limit (0.05 mg/L).

Although daphnids were the least acutely sensitive to TNB of the organisms tested, chronic toxicity data were generated in support of another project and are reported here.[22] The concentration data reported in Table 31 do not include reduced concentrations caused by toxicant diluter malfunctions on two separate days during the 21-day test. On days 0 and 1, a valve malfunction resulted in lowered toxicant concentrations at the top treatment level only. On day 4, only half the appropriate amount of toxicant was delivered to the test tanks over a 12-hour period.

Significant effects on daphnids were found at the top three concentrations (Table 32). In addition, some undeveloped eggs were shed from daphnids at these exposure levels. Daphnids at the 2.68 mg/L level showed a marked spinning motion when swimming. The no effect/effect range is 0.47 to 0.75 mg/L.

Early in the test, some daphnids at all concentration levels became caught in the surface film. There seems to be some concentration-dependency to this floating, although there is less at 1.32 mg/L than might be expected. Floating in the controls was due mostly to one of the four replicate tanks, which had 29 of the 38 floater days. Using the General Linear Models procedure on the Statistical Analysis System,[15] partial correlation coefficients were determined between the number of floater days and the other variables reported in Table 32. The only significant correlation was with daphnid length. Overall, the effect of floating on the outcome of the experiment is thought to be minimal. In any case, daphnids were substantially less sensitive to TNB chronic effects after a 21-day exposure than were the fathead minnows and rainbow trout exposed in ELS tests.

TABLE 27. RESULTS OF THE FIRST EARLY LIFE STAGE TEST WITH TNB AND RAINBOW TROUT

Mean Measured Concentration (mg/L)	Hatching Success (%)	Time to Hatch (days)[a]	Time to Swim-up (days)[a]	Fry Survival (%)	Overall Survival (%)	Fry Deformities (%)	Fry Length (mm)	Fry Weight (g)
0.71	86.2[b]	26	—	0[a]	0[a]	1.4	—	—
0.36	93.8	28	—	0[a]	0[a]	6.7	—	—
0.22	95.6	29	22	2.0[b]	1.9[b]	7.8	25.3[c]	0.237[c]
0.13	96.2	28.5	19.5	50.0[b]	48.1[b]	4.5	27.9[b]	0.342[b]
0.09	95.0	29	18	76.3	72.5	2.6	31.9[b]	0.503[b]
<0.05 (control)	95.6	29	17.5	77.8	74.4	8.5	36.0	0.676

a. Not analyzed statistically
b. Significantly different from the controls at the $p < 0.01$ level.
c. Not statistically evaluated; sample size (n=3) too small.

TABLE 28. MEASURED TNB CONCENTRATIONS DURING THE SECOND RAINBOW TROUT EARLY LIFE STAGE TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
0.200	0.174	0.011	0.154-0.190	10
0.113	0.082	0.011	0.065-0.099	10
0.065	0.045	0.007	0.037-0.054	9
0.035	0.022	0.006	0.012-0.030	10
0.020	0.015	0.005	0.010-0.023	10
0	BDL[a]	--	BDL-0.015	12

a. BDL - below detection limit (0.01 mg/L).

As with DiNA and DNB, algae appear to be highly sensitive to the effects of TNB relative to the other organisms tested. TNB was also the most toxic of the three compounds to S. capricornutum. Growth was significantly reduced at all concentrations tested on both days 5 and 14 of the test (see Table 33). Concentrations from 1.18 to 17.3 mg/L TNB were algicidal, while the lower concentrations (0.10 and 0.17 mg/L) were algistatic, in that renewed growth occurred after exposed cells were transferred into fresh algal assay medium. The 0.96 mg/L treatment level was intermediate in response; growth in clean medium was present, but was not at a very high level. Additional testing will be required in order to define a no effect concentration for TNB to this algal species.

TABLE 29. RESULTS OF THE SECOND EARLY LIFE STAGE TEST WITH RAINBOW TROUT AND TNB

Mean Measured Concentration (mg/L)	Hatching Success	Time to Hatch (days)[a]	Time to Swim-up (days)[a]	Fry Survival (%)	Overall Survival (%)	Fry Deformities (%)	Fry Length (mm)	Fry Weight (g)
0.17	89.2	25.5	17.0	48.6[b]	43.3[b]	7.5	35.1[b]	0.69[b]
0.082	90.8	26.0	15.5	71.6	65.0	11.0	45.2	1.37
0.045	91.2	26.0	15.5	87.3	80.0	8.2	44.6	1.32
0.022	92.5	25.5	15.5	77.5	71.7	12.6	46.5	1.50
0.015	90.0	26.0	15.0	83.3	75.0	11.1	46.2	1.47
<0.010 (control)	90.0	25.0	16.0	80.6	72.5	4.6	45.4	1.46

a. Not analyzed statistically.
b. Significantly different from the controls at the $p < 0.05$ level.

TABLE 30. COMBINED RESULTS FROM TWO RAINBOW TROUT EARLY LIFE STAGE TESTS WITH TNB FOR END POINTS ANALYZED STATISTICALLY

End Point	Concentration Range, Highest No-Effect Level - Lowest Significant Effect Level (Percent Difference From Controls)	
	Test 1	Test 2
Hatching Success	0.36-0.71 mg/L (-2% to -10%)	>0.17 mg/L
Fry Survival	0.09-0.13 (-2% to -36%)	0.082-0.17 (-11% to -40%)
Overall Survival	0.09-0.13 (-3% to -35%)	0.082-0.17 (-10% to -40%)
Fry Length	<0.09 (-11%)	0.082-0.17 (0% to -23%)
Fry Weight	<0.09 (-26%)	0.082-0.17 (-6% to -53%)

TABLE 31. MEASURED TNB CONCENTRATIONS DURING A DAPHNID CHRONIC TEST

Nominal Concentration (mg/L)	Mean Measured Concentration (mg/L)	Standard Deviation	Range	n
2.97	2.68	0.260	2.41-3.09	8
1.68	1.32	0.153	1.05-1.59	8
0.96	0.75	0.138	0.53-0.89	8
0.54	0.47	0.114	0.34-0.69	8
0.29	0.24	0.076	0.17-0.42	8
0	BDL[a]	—	—	7

a. BDL - Below detection limit (0.025 mg/L)

TABLE 32. RESULTS OF A CHRONIC TOXICITY TEST WITH DAPHNIA MAGNA AND TNB

Mean Measured Concentration (mg/L)	Survival (%)	Young per Female per Reproductive Day	Mean Total Young Per Tank	Total Length (mm)	Floater Days[a]
2.68	70	1.43[b]	127[b]	3.58[b]	183
1.32	90	3.52[b]	412[b]	4.23[b]	37
0.75	97.5	5.53	723[b]	4.27[b]	108
0.47	97.5	5.63	742	4.62	33
0.24	100.	5.00	742	4.69	28
<0.025 (control)	97.5	6.19	842	4.88	38

a. Floater days - The sum of the number of FO daphnids observed caught in the surface film each day at each treatment level over the duration of the test. Not analyzed statistically.
b. Significantly different from the controls at the $p < 0.05$ level.

TABLE 33. EFFECTS OF TNB ON THE GROWTH OF SELENASTRUM CAPRICORNUTUM[a]

Initial Measured Concentration (mg/L)	Growth (cells/mL)[b] Day 5	Day 14
17.32[c]	25,796[d]	15,609[d]
9.11[c]	23,973[d]	9,156[d]
1.18[c]	21,627[d]	7,964[d]
0.89[c]	40,542[d]	169,279[d]
0.17[e]	79,546[d]	1,110,862[d]
0.10	198,587[d]	2,556,800[d]
Control	2,600,667	3,784,427

a. Data from Bailey.[3,5]
b. Mean of three replicates (four for controls).
c. Cells washed and resuspended in clean media on Day 5.
d. Significantly different from the controls at the $p < 0.05$ level.
e. Nominal concentration; actual initial measured concentration was 0.10 mg/L.

CONCLUSIONS AND RECOMMENDATIONS

The results of the toxicity tests conducted with DiNA, DNB, and TNB are summarized in Tables 34 through 36. Overall, TNB was the most toxic of the three compounds, with all tests but the daphnid static acute test having effect levels below 1 mg/L. Both DiNA and DNB showed wide ranges of acute toxicity (about one order of magnitude) for the species tested.

The lowest no effect/effect ranges for the species tested are as follows by compound: DiNA - 0.03 to 0.13 mg/L; DNB - 0.16 to 0.42 mg/L; and TNB - 0.08 to 0.12 mg/L. It is important to note that a lower threshold for toxicity was not established for S. capricornutum and TNB, but is less than 0.10 mg/L. Assuming that species of aquatic organisms in field situations are about as sensitive to these compounds as the laboratory test organisms used in this study, these ranges give a rough idea of the concentrations of DiNA, DNB, and TNB which, if exceeded, may result in damage to aquatic communities. Calculation of formal water quality criteria, as defined by the US Environmental Protection Agency,[23] cannot be done without additional toxicity testing.

Further research should be undertaken to define the toxicity of TNB to S. capricornutum. In view of the sensitivity of this and other algal species to the nitroaromatic compounds evaluated in this study, it is important that algal species be included in future hazard evaluations with this type of material. Since many of these compounds are subject to photolytic decomposition, it would be useful to utilize or develop a chemostat-type device to allow algal toxicity tests to be performed with renewal of toxicant solutions with those compounds where photolysis may be a problem.

Both TNB and DiNA are formed during the photolysis of TNT.[1] Although studies have shown that TNT photolysis reduces its toxicity to aquatic organisms,[4] the formation of TNB and DiNA, which have been found in this study to cause detrimental effects at concentrations of 0.1 mg/L or below, could create a potentially hazardous situation. It would be advisable to evaluate the rates of TNB and DiNA formation in areas where TNT discharges occur.

TABLE 34. TOXICITY OF DINA TO AQUATIC ORGANISMS

Species[a]	Test End Point	Length of Exposure (days)	Test Type	Results (95% confidence limits, if available) (mg/L)
Fathead Minnow	LC50	4	Static Acute	21.1 (15.1-29.9)
Channel Catfish	LC50	4	Static Acute	13.9 (11.0-18.4)
Daphnia magna	EC50	2	Static Acute	13.8 (12.9-14.8)
Bluegill	LC50	4	Static Acute	7.0 (3.9-14.4)
Rainbow Trout	LC50	4	Static Acute	3.0 (2.1-3.8)
Daphnia magna	No Effect-Effect Range[b]	21	Chronic	2.41-4.56
Rainbow Trout	LC50	11	Flow-through LC50	2.0 (1.7-2.3)
	No Effect-Effect Range	71	Early Life Stage	0.37-0.65
Selenastrum capricornutum	No Effect-Effect Range[c]	5	Static Acute	0.03-0.13

a. Tests arranged from highest to lowest end point concentrations.
b. Range from the highest no-effect concentration to the lowest concentration tested causing inhibitory effects.
c. Based on growth inhibition; concentrations of 0.13-15.13 had algistatic effects.

TABLE 35. TOXICITY OF DNP TO AQUATIC ORGANISMS

Species[a]	Test End Point	Length of Exposure (days)	Test Type	Results (95% confidence limits, if available) (mg/L)
Selenastrum capricornutum	No Effect-Effect Range[b,c]	5	Static Acute	14.3-85.6
Daphnia magna	EC50	2	Static Acute	27.4 (24.0-31.0)
Fathead Minnow	LC50	4	Static Acute	16.8 (11.4- +∞)
Channel Catfish	LC50	4	Static Acute	8.1 (7.1-9.3)
Rainbow Trout	LC50	4	Static Acute	1.7 (1.4-2.4)
Bluegill	LC50	4	Static Acute	1.4 (1.2-2.3)
Rainbow Trout	No Effect-Effect Range	69	Early Life Stage	0.50-0.97
Selenastrum capricornutum	No Effect-Effect Range[d]	5	Static Acute	0.26-0.97
Rainbow Trout	No Effect-Effect Range (mortality only)	30	Flow-through LC50	0.16-0.42

a. Tests arranged from highest to lowest end point concentrations.
b. Range from the highest no-effect concentration to the lowest concentration tested causing inhibitory effects.
c. Based on algicidal effects.
d. Based on growth inhibition; concentrations of 10.72 and 14.33 mg/L were algistatic.

TABLE 36. TOXICITY OF TNB TO AQUATIC ORGANISMS

Species[a]	Test End Point	Length of Exposure (days)	Test Type	Results (95% confidence limits, if available) (mg/L)
Daphnia magna	EC50	2	Static Acute	4.1 (2.6-7.7)
Selenastrum capricornutum	No Effect-Effect Range[b,c]	5	Static Acute	0.96-1.18
Bluegill	LC50	4	Static Acute	0.85 (0.52-1.38)
Daphnia magna	No Effect-Effect Range	21	Chronic	0.47-0.75
Rainbow Trout	LC50	4	Static Acute	0.52 (0.32-0.80)
Fathead Minnow	LC50	4	Static Acute	0.49 (0.44-0.56)
	LC50	10	Flow-through LC50	0.46 (0.42-0.53)
Rainbow Trout	LC50	18	Flow-through LC50	0.43 (0.24-0.73)
Channel Catfish	LC50	4	Static Acute	0.38 (0.34-0.43)
Rainbow Trout	No Effect-Effect Range	71	Early Life Stage	0.08-0.17
Fathead Minnow	No Effect-Effect Range	32	Early Life Stage	0.08-0.12
Selenastrum capricornutum	Growth Inhibition	5	Static Acute	<0.10[d]

a. Tests arranged from highest to lowest end point concentrations.
b. Range from the highest no-effect concentration to the lowest concentration causing inhibitory effects.
c. Based on algicidal effects.
d. Significant difference from the controls noted at the lowest concentration tested.

LITERATURE CITED

1. Burlinson, N.E. 1980. Fate of TNT in an Aquatic Environment: Photodecomposition vs. Biotransformation. Report NSWC TR 79-445. Naval Surface Weapons Center, Silver Spring, MD.

2. Liu, D.H.W., R.J. Spanggord, H.C. Bailey, H. S. Javitz, and D.C.L. Jones. 1983. Toxicity of TNT Wastewaters to Aquatic Organisms. Volume II. Acute Toxicity of Condensate Wastewater and 2,4-Dinitrotoluene. Draft final report. SRI, International, Menlo Park, CA. DAMD17-75-C-5056.

3. Bailey, H.C. 1983. Personal communication.

4. Liu, D.H.W., R.J. Spanggord, H.C. Bailey, H. S. Javitz, and D.C.L. Jones. 1983. Toxicity of TNT Wastewaters to Aquatic Organisms. Volume I. Acute Toxicity of LAP Wastewater and 2,4,6-Trinitrotoluene to Aquatic Organisms. Draft final report. SRI, International, Menlo Park, CA. DAMD17-75-C-5056.

5. Bailey, H.C. 1982. Toxicity of TNT wastewater to aquatic organisms. Monthly Progress Report No. 80. SRI International, Menlo Park, CA. DAMD17-75-C-5056.

6. Bringmann, G. and R. Kuehn. 1978. Testing of substances for their toxicity threshold: model organisms Microcystis aeruginosa and Scenedesmus quadricauda. Mitt. Int. Ver. Theor. Angew. Limnol. 21:275-284. In: Wentsel, R.S., R.G. Hyde, W.E. Jones, III, M.J. Wilkinson, W.E. Harward, III, and J.F. Kitchens. 1979. Problem Definition Study on 1,3-Dinitrobenzene, 1,3,5-Trinitrobenzene, and Di-N-propyl Adipate. Final report. Atlantic Research Corporation, Alexandria, VA. DAMD17-77-C-7057.

7. Bringmann, G. and R. Kuehn. 1980. Comparison of the toxicity thresholds of water pollutants to bacteria, algae, and protozoa in the cell multiplication inhibition test. Water Res. 14:231-241.

8. LeClerc, E. 1960. The self-purification of streams and the relationship between chemical and biological tests. Proc. 2nd Symp. on Treatment of Waste Waters, 281-316. In: Wentsel, R.S., R.G. Hyde, W.E. Jones, III, M.J. Wilkinson, W.E. Harward, III, and J.F. Kitchens. 1979. Problem Definition Study on 1,3-Dinitrobenzene, 1,3,5-Trinitrobenzene, and Di-N-Propyl Adipate. Final report. Atlantic Research Corporation, Alexandria, VA. DAMD17-77-C-7057.

9. Juhnke, V.I. and D. Ludemann. 1978. Results of the study of 200 chemical compounds on acute fish toxicity using the golden orfe test. A. Wasser Abwasser Forsch. 11(5):161-164. In: Wentsel, R.S., R.G. Hyde, W.E. Jones, III, M.J. Wilkinson, W.E. Harward, III, and J.F. Kitchens. 1979. Problem Definition Study on 1,3-Dinitrobenzene, 1,3,5-Trinitrobenzene, and Di-N-Propyl Adipate. Final report. Atlantic Research Corporation, Alexandria, VA. DAMD17-77-C-7057.

10. Curtis, M.W. and C.H. Ward. 1981. Aquatic toxicity of 40 industrial chemicals - testing in support of hazardous substances spill prevention regulation. J. Hydrol. 51:359. In: Spehar, R.L., G.M. Christensen, C. Curtis, A.E. Lemke, T.J. Norberg, and Q.H. Pickering. 1982. Effects of pollution on freshwater fish. J. Water Pollut. Control Fed. 54(6):877-922.

11. Goulden, C.E, R.M. Comotto, J.A. Hendrickson, Jr., L.L. Hornig, and K.L. Johnson. 1982. Procedures and recommendations for the culture and use of Daphnia magna in bioassay studies. In: J.G. Pearson, R.B. Foster, and W.E. Bishop, eds. Aquatic Toxicology and Hazard Assessment: Fifth Conference. ASTM STP 766. American Society for Testing and Materials, Philadelphia, PA. pp. 139-160.

12. Lemke, A.E., W.A. Brungs, and B.J. Halligan. 1978. Manual for Construction and Operation of Toxicity-testing Proportional Diluters. EPA-600/3-78-072. US Environmental Protection Agency, Environmental Research Laboratory-Duluth, Duluth, MN.

13. Stephan, C.E. 1978. LC50 program. Unpublished data. US Environmental Protection Agency, Environmental Research Laboratory-Duluth, Duluth, MN.

14. Feder, P. 1980. Design and Analysis of Chronic Aquatic Tests of Toxicity. Draft final report. Battelle Columbus Laboratories, Columbus, OH. DAMD17-79-C-9150.

15. Statistical Analysis System. 1979. SAS Institute, Raleigh, NC.

16. Williams, D.A. 1971. A test for differences between treatment means when survival dose levels are compared with a zero dose control. Biometrics. 27:103-117.

17. Payne, W.G. and R.H. Hall. 1979. A method for measuring algal toxicity and its application to the safety assessment of new chemicals. In: L.L. Marking and R. A. Kimerle, eds. Aquatic Toxicology. ASTM STP 667. American Society for Testing and Materials, Philadelphia, PA. pp. 171-180.

18. American Society for Testing and Materials. 1980. Standard practice for conducting acute toxicity tests with fishes, macroinvertebrates, and amphibians. E 729-80. American Society for Testing and Materials, Philadelphia, PA.

19. Randomization patterns were obtained using an APL computer program written by P. Gibbs, US Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, Frederick, MD.

20. Schimmel, S. 1983. Proposed new standard practice for conducting toxicity tests with the early life-stages of fishes. Draft no. 5. American Society for Testing and Materials Subcommittee E-47.01, Philadelphia, PA.

21. McKim, J.M. 1977. Evaluation of tests with early life stages of fish for predicting long term toxicity. J. Fish Res. Bd. Can. 34:1148-1154.

22. van der Schalie, W.H. 1983. Unpublished data. US Army Medical Bioengineering Research and Development Laboratory, Fort Detrick, Frederick, MD.

23. US Environmental Protection Agency. 1980. Guidelines for deriving water quality criteria for the protection of aquatic life and its uses. Federal Register 45(231):79318 (November 23, 1980).

DISTRIBUTION LIST

No. of Copies	
5	US Army Medical Research and Development Command ATTN: SGRD-RMS Fort Detrick, Frederick, MD 21701
12	Defense Technical Information Center (DTIC) ATTN: DTIC-DDA Cameron Station Alexandria, VA 22314
1	Commandant Academy of Health Sciences, US Army ATTN: HSHA-DCD Fort Sam Houston, TX 78234
2	Library Technician US Army Medical Bioengineering Research and Development Laboratory ATTN: SGRD-UBZ-IL Fort Detrick, Frederick, MD 21701
1	Chief USAEHA Regional Division, West Fitzsimmons AMC Aurora, CO 80045
1	Chief USAEHA Regional Division, North Fort George G. Meade, MD 20755
1	Chief USAEHA Regional Division, South Bldg 180 Fort McPherson, GA 30330
1	Commander USA Health Services Command ATTN: HSPA-P Ft. Sam Houston, TX 78234
1	Commander USA Const. Engr. Research Laboratory P.O. Box 4005 Champaign, IL 61820
1	Commandant Academy of Health Sciences, US Army ATTN: HSHA-IPM Ft. Sam Houston, TX 78234

1 Commander
US Army Materiel Development and Readiness Command
ATTN: DRCSG
5001 Eisenhower Avenue
Alexandria, VA 22333

1 Department of the Army
Office of the Chief of Engineers
ATTN: DAEN-ZCE
Washington, DC 20310

1 HQDA (DASG-PSP-E)
WASH DC 20310

1 Commander
US Army Environmental Hygiene Agency
ATTN: HSHB-E
Aberdeen Proving Ground, MD 21010

1 US Army Corps of Engineers
Huntsville Division
ATTN: EP-PM
P.O. Box 1600
Huntsville, AL 35807

1 Commander
US Army Environmental Hygiene Agency
ATTN: Librarian, HSHD-AD-L
Aberdeen Proving Ground, MD 21010

1 Commander
US Army Armament Research & Development Center
ATTN: DRSMC-LCM-SA (D)/Mr. J.M. Swotinsky
Dover, NJ 07801

1 Commander
US Army Natick Research and Development Command
ATTN: DRDNA-YE
Natick, MA 01760

1 Commander
US Army Toxic and Hazardous Materials Agency
ATTN: DRXTH-TE-D
Aberdeen Proving Ground, MD 21010

5 Commander
US Army Toxic and Hazardous Materials Agency
ATTN: DRXTH-ES
Aberdeen Proving Ground, MD 21010

1 Dean
School of Medicine
Uniformed Services University of the Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20014

1 Mr. W. Barnes
National Fisheries Center
P.O. Box 700
Kearneysville, WV 25430